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DOI: https://doi.org/10.1016/j.physbeh.2014.02.060

Growing up in the family or growing up alone influences behavior and hormones, but not arginine vasopressin receptor 1a expression in male African striped mice

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Abstract

In many species males display alternative reproductive tactics (ARTs). While males of different tactics differ behaviorally in the field, it is often not known whether these behavioral differences would also occur under standardized laboratory conditions, nor how ARTs are regulated by the brain. In the present study we kept male African striped mice (*Rhabdomys pumilio*) in captivity either in family groups or solitary, to mimic ARTs observed in the field. This allowed us to study these males behaviorally under standardized conditions, to replicate physiological findings from the field, and to study the expression of the arginine vasopressin 1a receptor (AVPR1a) in their brains. Changes in either peptide release or receptor expression (or both) might regulate ARTs with differential timelines, with peptide secretion being faster than receptor expression. As observed in the field, family living males had higher corticosterone but lower testosterone levels than singly housed males. Surprisingly, singly housed males were less aggressive while at the same time having higher testosterone levels. We found no differences in AVPR1a expression. In a previous study it was shown that singly housed males have higher levels of AVP stored in their brain, which potentially could be secreted when the social situation changes, for example to establish social bonds. Our study on AVPR1a suggests the hypothesis that, given that the receptor distribution and expression of singly housed males does not differ from that of family-living males, the brains of singly-housed males have a similar capacity to be responsive to AVP when given the chance to interact socially.

Keywords: paternal care, helper, communal breeding, intra-specific variation, social organization, social flexibility

1. Introduction

In many species, males display alternative reproductive tactics (ARTs) to optimize their individual reproductive success [1]. In some species these tactics are genetically determined and fixed for life, In other species with plastic tactics males can change from one tactic to another depending on their own condition (age, body mass) and on environmental conditions [1, 2]. However, from a proximate point of view it is not well understood which behavioral, hormonal and neuroendocrine changes are associated with tactic changes.

In species with plastic ARTs, it is difficult to distinguish in how far the behavioral differences between tactics represent internal motivational difference between males, or are simply triggered by differences in environmental stimuli. An example of externally induced behavioral differences would be if males of the dominant tactic (also called bourgeois tactic) might be more aggressive because they are typically larger than the other males they encounter, while sneaker males might be less aggressive only because their opponents are larger than themselves. It is important to test males of different tactics under standardized conditions; for example, always with an opponent male that is smaller than the focal male.

It is well known that males following differing ARTs may differ in their hormonal profiles. Dominant males typically having higher androgen levels [2, 3] and lower glucocorticoid levels [2]. In contrast to steroids, we know very little about the role of neuropeptides in the regulation of ARTs. The neuropeptide arginine vasopressin (AVP) modulates social behaviors by activating its receptor 1a (AVPR1a) [4, 5]. Variation in this receptor has been shown in brain areas of the “social behavior network” (lateral septum, amygdala, ventral pallidum) [6] which are known to regulate social recognition and pair bond formation [7, 8]. Experimentally induced

expression of AVPR1a in laboratory mice increased pro-social behaviors [5]. It has been suggested that variation in the expression of AVPR1a explains a significant amount of variation in social behavior of voles of the genus *Microtus*, both within [4, 9] and between species [10]. While there is good evidence for an important role of AVPR1a in the regulation of social behavior in laboratory rodents, little is known about its role in the regulation of ARTs, with the exception of one field study showing differences in AVPR1a expression in brain areas involved in spatial memory between territorial males and wanderers of the prairie vole (*Microtus ochrogaster*) [9] but not in brain areas suggested to be important for pair-bonding in prairie voles [32, 33]. ARTs are typically studied in the field and not in the laboratory. To study AVPR1a expression one has to sacrifice the animals and collect the brains, which is typically not an option in field studies, as this would greatly affect the entire study population. Therefore, it is important to be able to study ARTs in captivity, where such samples can be collected.

One species with male ARTs that can be studied both in the field and in captivity is the African striped mouse (*Rhabdomys pumilio*). Male striped mice have three alternative reproductive tactics that differ in their hormone levels [11] and in reproductive success [12]: 1. Philopatric males remain in their family group after reaching adulthood. They have very low reproductive success (with neighboring females), low testosterone and high corticosterone levels. Philopatric males typically switch to another tactic when they become heavier. 2. Solitary roamers leave their family group and live alone. They have very high testosterone and low corticosterone levels and typically low reproductive success. They might later immigrate into a group of communally breeding females and become 3. territorial breeding males with somewhat lower testosterone levels, low corticosterone levels and high reproductive

success. Males can switch between tactics [11] and their hormonal profiles change as they do so [13]. If population density is very high, most males remain as adult philopatrics in their family group, but when population density is very low, many males become solitary roamers when reaching puberty [14]. Thus, within the same species and same population, males might either grow up in extended family groups or become solitary, representing two very different social pathways.

These tactics as observed in the field have been mimicked in captivity by separating brothers at an age of 3 weeks [14]. One brother remained in the family, representing the philopatric tactic, while his brother was housed singly, representing the roaming tactic. This leads to predicted physiological differences, with singly housed males showing lower corticosterone and higher testosterone levels, becoming sexually mature at an earlier age, developing larger testes and producing more sperm [15-17]. In the present study we used the same experimental approach and we had three aims. (I) We compared the social behavior of family versus singly housed males, to determine whether males following simulated ARTs differ in social behavior when tested under identical standardized conditions. We predicted that family housed males would show more aggression towards strange males, because families defend territories while roamers do not [11, 18, 19]. We further predicted that singly housed males would show more pro-social behaviors towards strange females than family-housed males, as roamers would be more ready to mate. (II) We measured steroid hormone levels and predicted singly house males to have higher testosterone and lower corticosterone levels than their family housed brothers. (III) We measured AVPR1a in the brains of males using all three tactics to examine whether differences in AVPR1a expression are associated with different housing conditions (social versus solitary).

2. Materials and Methods

2.1.Animals

The colony consisted of animals originally trapped in 2002 in the Succulent Karoo (Goegap Nature Reserve) in South Africa. Animals were bred at the research station in Goegap under natural weather conditions and F10 descendants were exported from there to the University of Zurich in October 2006 where breeding continued. Animal ethical clearance for the experiments was provided by the Kantonale Veterinärmt of the Kanton Zürich in Switzerland (ethical clearance number 91/2006).

2.2.Housing conditions

The study took place from November 2006 to June 2007. Animals were housed at the University of Zurich under a 11.5:12.5h light regime. Wild rodents kept in captivity are prone to develop stereotypic behavior (for striped mice see [20]) which is known to both affect social behavior as well as physiology and brain structure [21]. Thus, all animals were housed under super-enriched conditions which were successful in avoiding the development of stereotypic behavior (recorded during 15min observations per individual): none of the 16 family housed males and none of the 16 singly housed males showed any stereotypic behaviors (see below for details). Wheel running was observed in 6 family-housed and in 4 singly-housed males and did not differ between the two treatments (82.7 $\pm$ 146.3s *vs.* 55.8 $\pm$ 116.6s respectively; T=9, W=18, Wilcoxon-test; p=0.25).

Pairs and families were housed in two 50x30x30cm glass tanks which were connected to one another by a flexible plastic tube. A second tube was connected to one 20x13x15cm plastic cage (type 4 cage) which contained a water bottle. Single

individuals were housed in one glass tank connected to two plastic cages. All tanks and cages had 5cm of wood shavings as bedding. The tanks additionally contained natural branches for enrichment. Furthermore, each family and each singly housed mouse had one running wheel, because we found in a pilot study that running wheels reduce stereotypic behavior.

Each family and each singly housed mouse had access each week for 1-2 days to an extra 70x50x35cm enriched tank. Connection was done by removing one type 4 cage and replacing it with another type 4 cage which was connected by flexible tubes to the home tank as well as to the large enriched tank. The large enriched tank was provided with bedding, tubes and branches. Since up to five families and single mice had access to one largeenriched tank on different days, mice could directly experience olfactory cues from unrelated/unfamiliar mice in these large enriched tanks.

Mice were supplied with water *ad libitum*. Striped mice in the Succulent Karoo gain a lot of weight during spring and lose more than 10% during the following dry season [22]. This might be the reason why they are very prone to extreme obesity in captivity. To avoid obesity and as a means of behavioral enrichment, mice were not fed *ad libitum,* but on the following schedule: in the morning they received a seed mix of 4.0g/individual (guinea pig and hamster food, Haefliger AG, Herzogenbuchsee, Switzerland), at noon one piece (approx. 1.0g) of fruit or vegetable per individual and in the afternoon two mealworms per individual.

2.3.Experimental procedure

Families were housed together until offspring were three weeks old (D21) and thus 5 days after weaning (which is on D16) [38]. At this time, only one male and one

female offspring remained with each pair. These males were the focal individuals of the *family* treatment. They remained with their parents and all experienced the raising of the next litter. To avoid crowding in family cages, all juveniles except one male and one female of the second litter were removed when they reached 3 weeks of age. No pair had a third litter within the study period of 10 weeks.

From the juveniles that were removed at an age of 3 weeks, one male from each litter was housed singly as described above. We therefore followed a paired design with one brother being family housed, the other brother singly housed. Brothers were randomly assigned to treatments.

Each mouse from the family and single treatment was weighed once per week and its reproductive state was determined until both males were categorized as being fully scrotal (testes descended into the scrotum, indicating sexual maturity). When there was a difference between siblings in the week the first male became scrotal, a blood sample was taken to compare testosterone and corticosterone levels to test whether the difference in reaching sexual maturity was associated with endocrine differences. Blood samples were taken the day after the difference was found early in the morning (controlling for circadian rhythms of hormone secretion), to avoid a stress response due to handling during inspection. Samples were then obtained within three minutes: mice were anaesthetized using Methoxyfluran and a blood sample of 100-300µl was taken from a sublingual tongue vein [23]. Blood samples stood at room temperature for 1.5h and were then centrifuged for 10min. The resulting serum was pipetted and frozen in aliquots of 20ul for corticosterone and 50ul for testosterone.

2.4.Behavioral testing at age of 10 weeks

Two encounter tests were conducted on two successive days to compare social behavior under standardized conditions (prediction 1 from the introduction). One test was a same-sex encounter, the other one an opposite-sex encounter. For both brothers, the same kind of test was always conducted on the same day. For half of the males, the first day a same-sex and the second day an opposite-sex encounter test was conducted, for the other males the order was reversed.

Stimulus animals in these tests were all adults housed in sibling groups from the same breeding colony. In all cases, the stimulus animal weighed less than the test animal (10.2 $\pm$ 1.3g less than family housed males and 9.2 $\pm$ 1.0g less than singly housed males), because dominance is related to weight in striped mice, independent of sex [18]. Thus, the test animal had the opportunity to dominate the other one and initiate aggression or pro-social behaviors, such that results represented the motivation of the test animal, not the stimulus animal.

All tests were performed in a neutral 80x40x60cm arena made of wood. At the beginning of the test, a partition in the middle divided the arena in two compartments. The stimulus animal was placed in one compartment, while the test animal was placed in the other compartment. After a habituation period of 5 min, the partition was removed and the test animal was observed as focal animal for 15 min. No damaging fights occurred that would have made it necessary to terminate observations earlier. The frequency of aggressive behaviors (fight, bite, chasing) was recorded as well as of grooming the stimulus animal by the test animal. The total time spent in bodily contact was also recorded.

2.5.Brain and blood sampling at age of 10 weeks

Two days after the last encounter study, males were anaesthetized with Methoxyfluran and decapitated early in the morning (within 1 hour after lights were on), controlling for circadian rhythms of hormone secretion. Trunk blood was collected and processed as described above. Brains were removed and rapidly frozen on crushed dry ice. Brains were then wrapped in aluminum foil and stored at -80ºC.

2.6.Hormone assays

Commercial kits from IBL Hamburg were used for both hormone assays. Procedures were as stated in the kits manuals. However, due to very high corticosterone levels, samples were diluted 1:99. In some cases samples for testosterone measurements were too small and had to be diluted 1:1 with the zero standard. All measurements were well within the standard curve of the assay.

For both hormones, serial dilution of striped mouse sample pools (2 for each hormone) paralleled the standard curve and the slopes were not different [24]. Intra- and inter-assay variability was determined with pools from wild striped mice. Eight measurements were conducted for intra-assay, five for inter-assay variability. For corticosterone, intra-assay variability was 8.3. Inter-assay variability was 6.4 and 2.3%. For testosterone, intra-assay variability was 12.89 and 6.9%. Inter-assay variability was 19.1 and 9.9%.

2.7.Brain tissue preparation and autoradiography

Brain tissue was collected and flash frozen, and subsequently shipped to the University of California, Davis (United States) on dry ice. Six sets of coronal sections (20 microns thick; 100 microns apart) were cut on a Leica CM3050S cryostat between

-18 and -22º C starting anterior of the corpus callosum and extending caudally to the posterior hippocampus. Sections were thaw mounted onto Superfrost plus slides (Fisher Scientific) and stored at -80º C until autoradiographical assay.

Autoradiography was conducted using a 50 µCi/ml concentration of (d(CH2)51,Tyr(Me)2,Arg8)-Vasopressin (Bachem America) following standard protocols [25]. In brief, thawed sections were fixed in 1% paraformaldehyde for 10 minutes, washed twice in Tris buffer for 10 minutes, and incubated for 60 minutes in I^{125}labeled ligand in 50ml Tris, 1mg $MgCl_2$, and 0.5g bovine serum albumin (BSA). Excess ligand was removed by four 5-minute washes in 4º C Tris $MgCl_2$. Slides were rocked in Tris $MgCl_2$ for 30 minutes at room temperature, dipped in 4º C distilled water and air dried. Nonspecific binding was determined by incubating adjacent sections with a 50 µCi/ml concentration of a specific V1a antagonist (Phenylacetyl1, 0-Me-D-Tyr2, [^{125}I-Arg6]-) as a displacer. This ligand displaced receptor binding to background levels, verifying the specificity of the ligand under the conditions used. Sections were exposed to Kodak BioMax MR film, and developed using Kodak GBX fixer and developer.

Structures were identified using the mouse brain atlas. Optical density measures for receptor binding were made using the NIH Image J program. Each region of interest was identified and measured bilaterally throughout each structure. In addition, a single slice in each region of each brain was identified at the depth of maximum binding for that structure. Non-specific binding for each slice was taken from a nearby region not expected to have vasopressin receptors. Specific binding was calculated by subtracting nonspecific binding from total binding in each area of interest. Receptor binding was quantified in five brain regions (Fig. 1): the posterior cingulate cortex (PCC), medial amygdala (MeA), ventral pallidum (VP), and lateral

septum (LS), as well as an area identified as the magnocellular preoptic nucleus (MCPO) based on the mouse brain atlas. These areas were chosen because of associations with mating-induced aggression (LS); social behavior, social memory and social motivation (MeA, VP); or space use and territoriality (PCC) [7]. The MCPO was quantified because of particularly dark binding in that area in many samples.

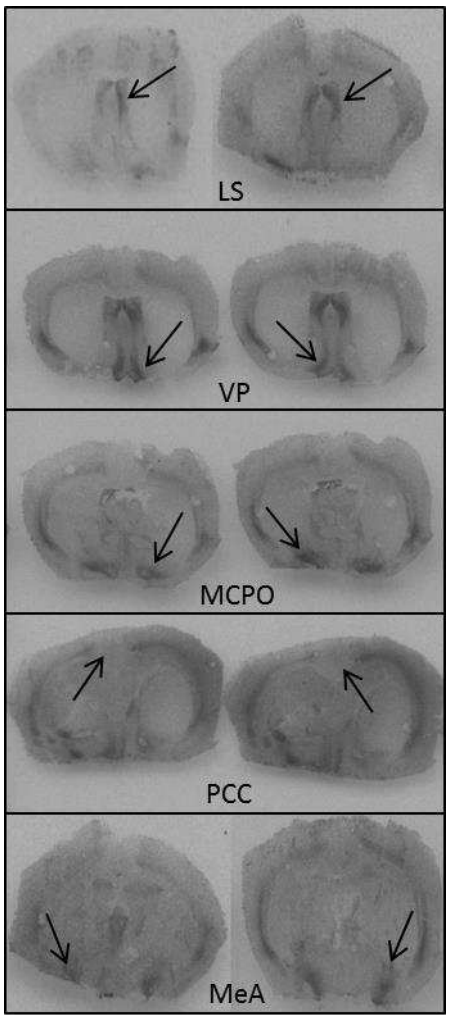


Figure 1. Arginine vasopressin receptor 1a binding in different brain areas of striped mice. LS: lateral septum; VP: ventral pallidum; MCPO: magnocellular preoptic nucleus; PCC: posterior cingulate cortex ; MeA: medial amygdale.

Table 1:
Sample sizes for the different comparisons. In each of the 22 families we measured age and body mass when becoming scrotal. Males were younger when we took the first blood sample and we could not repeat measurements when we got bad double values(>10%), which is why for some males only corticosterone values are available, from others only testosterone. We only collected the first blood sample when we had a difference in scrotality (no diff scrotality: no blood sample was collected). The second blood sample was collected from all remaining 16 pairs. Seven pairs were omitted from the experiment due to the death of three control males for unknown reasons and because four experimental male were rejected from their family and had to be separated (mentioned under "Note"). As we aimed for a paired data design, the other male then did not enter the study either. "Paired blood" refers to the comparison within individuals from blood 1 to blood 2 (Fig, 3). In addition, we sampled brains from 8 breeding males from additional families (not shown in table).

Family	Note	Scrotality	Blood 1	Blood 2	Paired blood	Brain	Behavior
4	Control male died	yes	(no diff. scrotality)				
6	Control male died	yes	both				
7		yes	both	both	both	yes	yes
9		yes	(no diff. scrotality)	both		yes	yes
10	Test male rejected	yes	both				
12		yes	Cort	both	Cort	yes	yes
17		yes	Testo	both	Testo	yes	yes
18		yes	both	both	both	yes	yes
19		yes	(no diff. scrotality)	both		yes	yes
21	Control male died	yes	both				
25	Test male rejected	yes	both				
32		yes	(no diff. scrotality)	both		yes	yes
34	Test male rejected	yes	both				
35		yes	both	both	both	yes	yes
38		yes	Testo	both	Testo	yes	yes
44		yes	Cort	both	Cort	yes	yes
46		yes	(no diff. scrotality)	both		yes	yes
47		yes	both	both	both		yes
66	Test male rejected	yes	both				yes
68		yes	Testo	both	Testo	yes	yes
70		yes	(no diff. scrotality)	both		yes	yes
77		yes	both	both	both	yes	yes
Total N		**22**	**13/14**	**15**	**7/8**	**15**	**16**

2.8.Statistics

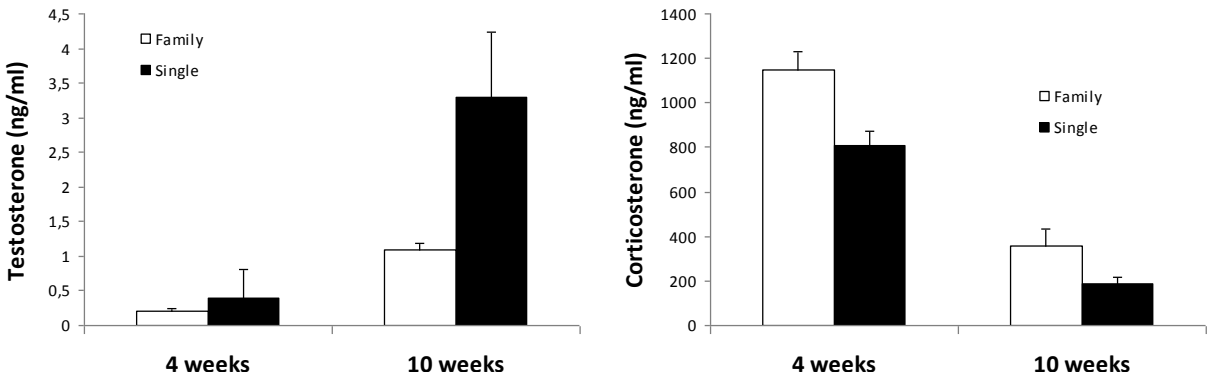
Sample sizes for the different comparisons are shown in Tab. 1. In total, 22 families took part in the study, but sample sizes for the different comparisons differ for several reasons (Tab. 1). For comparisons when males became scrotal, males from all 22 families were used. In 6 families, both males became scrotal the same week. As we were interested in how males differ in hormone levels when they differ in the time they became scrotal, we did not collect blood samples from males of these 6 families.

As males were small, only a small amount of blood could be collected and samples with bad double values could not be repeated, such that effective sample size for corticosterone measurements was 13 and for testosterone 14. Seven families were omitted from the experiment as three control males died for unknown reasons and four experimental males were rejected from their family and had to be separated. At the end of the experiment, we collected behavioral data from all remaining 16 families and blood samples from 15. From males of 7 families, corticosterone measurements were available for both periods (week both males were scrotal and end of experiments) and for males from 8 families for testosterone. We obtained brain samples from both the family and the singly housed males from the same 15 families. Additionally, we collected the brains from the breeding males from 8 additional families kept in the identical way. These samples (from pairs 65, 66, 76, 78, 80, 82, 88, 92) were collected after the other experiments were finished, as we decided then to include brains of male breeders for comparison as we had new field data indicating that considering this third tactic would be important [11]. Not all regions of all brains were measured, because some tissue was lost during the assay and because of partial thawing during courier transport. Sample sizes for family/solitary/breeding males were for lateral septum 13/10/8; for ventral pallidum 13/10/6, for MCPO 6/9/5, for PCC 15/13/8 and for MeA 13/14/8. As a consequence, we had empty cells and could not run comprehensive models but used the non-parametric Kruskal-Wallis Test (results would have been the same with one way ANOVA). As data were often not normally distributed, we used non-parametric statistics, always two-tailed. Paired comparisons (between the two brothers or for the same males tested twice) were done using the Wilcoxon-matched pairs rank sign test. Tests were done using Instat 3.05. Data are presented as mean $\pm$ SE.

Results

2.9.Comparison of body mass

At D21, when the experiment started, body mass of singly housed males (14.9 ± 2.9g) did not differ from the body mass of their family housed brothers (15.1 ± 2.9g; T=85, W=61, N=21, Wilcoxon test, p=0.30). Singly housed males were scrotal at a significantly younger age than family housed males (4.3 ± 0.5 weeks *vs.* 6.1 ± 1.8 weeks; Wilcoxon test, T=0, W=120, N=22, p=0.0002). Singly housed males had a significantly lower body mass when they became scrotal (26.1 ± 4.6g) than their family-living brothers (32.8 ± 7.9g; T=34, W=163, Wilcoxon test, p<0.01). At the end of the experiments, body mass of singly housed males (48.9 ± 6.3g) did not differ from the body mass of their family housed brothers (48.7 ± 6.1g; T=66.5, W=3, N=16, Wilcoxon test, p=0.94).



a) b)

Figure 2. Testosterone (a) and corticosterone (b) levels in family and singly housed males when approx. 4 weeks old (the first brother became scrotal) and 10 weeks old (all males were scrotal). Family housed males had significantly higher corticosterone and significantly lower testosterone levels than singly housed males during both periods.

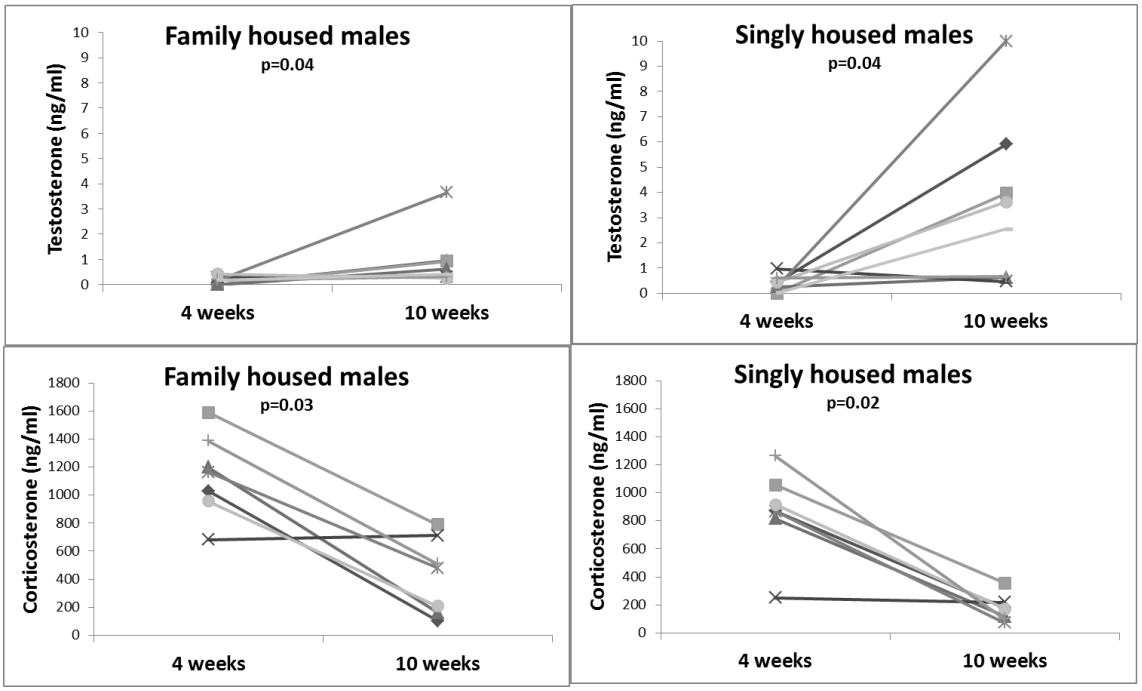


Figure 3. Changes in hormone levels of family housed males (left) and singly housed males (right) for testosterone (top; N=8 males for each category) and corticosterone (bottom; N=7 males for each category). The values of each individual are connected by a line (paired data).

1.1.Endocrine differences between singly housed, scrotal males; and family-housed brothers which were not yet scrotal (4-5 weeks old)

Scrotal singly housed males had significantly higher testosterone levels (0.42 $\pm$ 0.31 ng/ml) than their non-scrotal family housed brothers (0.15 $\pm$ 0.15 ng/ml) (T=3, W=72, Wilcoxon-test, p=0.002; Fig. 2a), and significantly lower corticosterone levels (807 $\pm$ 267ng/ml *vs.* 1146 $\pm$ 299 ng/ml; T=0, W=91, Wilcoxon-Test, p=0.0002; Fig. 2b) the week they became scrotal.

1.2.Endocrine differences at 10 weeks (end of experiment, all males scrotal)

At the age of 10 weeks at the end of the experiment, when all males were scrotal, singly housed males had significantly higher testosterone levels (3.262 $\pm$ 3.64 ng/ml) than their family housed brothers (1.08 $\pm$ 1.57 ng/ml; T=16, W=88, Wilcoxon-test,

p=0.01; Fig. 2a) and significantly lower corticosterone levels (187 ± 124 ng/ml *vs.* 359 ± 248 ng/ml; T=22, W=76, Wilcoxon-Test, p=0.03; Fig. 2b).

Testosterone levels of family housed males significantly increased during the course of the study from 0.16 ± 0.15 ng/ml (the week their brother became scrotal) to 0.93 ± 1.14 ng/ml at an age of 10 weeks (N=8, T=3, W=30, p=0.04; Fig. 3a). At the same time their corticosterone levels significantly decreased from 1144 ± 295 ng/ml to 424 ± 273 ng/ml (N=7, T=1, W=26, p=0.03; Fig 3b). The same changes were observed for singly housed males: their testosterone levels increased significantly (from 0.40 ± 0.23 ng/ml to 3.48 ± 3.27 ng/ml; N=8, T=3, W=30, p=0.04; Fig 3c) and their corticosterone levels decreased significantly (from 863 ± 310 ng/ml to 174 ± 95 ng/ml; N=7, T=0, W=28, p=0.02; Fig. 3d).

1.3.Behavioral differences at an age of 10 weeks

1.3.1. Social behaviors towards other males:

Singly housed males groomed the stimulus males significantly more often than did family housed males (4.6 ± 6.3 times/300s *vs.* 0.8 ± 1.7 times /300s repectively; T=4, W=70, Wilcoxon-test, p=0.003; Fig. 4), they spent significantly more time in bodily contact with them (216 ± 268s /300s *vs.* 36 ± 91s /300s; T=4.5, W=69, Wilcoxon-test, p=0.003; Fig. 4), and they showed significantly less aggression towards them (3.6 ± 7.4 times/300s *vs.* 9.3 ± 8.5 times /300s respectively; T=18.5, W=68, Wilcoxon-test, p=0.03; Fig. 4).

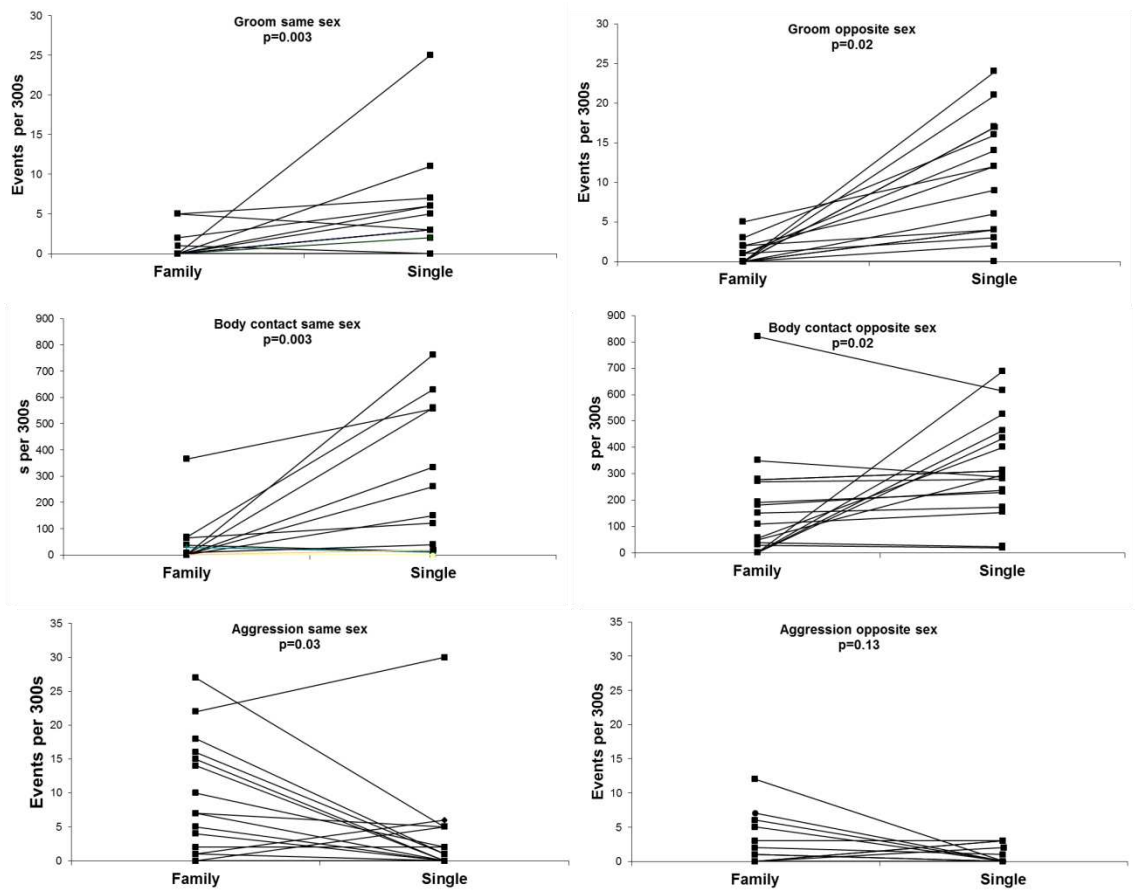


Figure 4. Behavioral differences between family housed males and their singly housed brothers. The data for each of the 16 brother pair are connected by a line. Left, same sex: behaviors shown towards strange males in a neutral presentation arena. Right, opposite sex: the same behaviors shown towards strange females.

1.1.1. Social behaviors towards females:

Singly housed males groomed the stimulus females significantly more often than did family housed males (10.3 $\pm$ 7.4 times/300s *vs.* 0.9 $\pm$ 1.4 times /300s respectively; T=0, W=120, Wilcoxon-test, $p<0.0001$; Fig. 4), and they spent significantly more time in bodily contact with them (321 $\pm$ 192s /300s *vs.* 158 $\pm$ 210s /300s; T=24, W=88, Wilcoxon-test, p=0.02; Fig. 4). There was no significant difference in the frequency of aggressive interactions with females (0.6 $\pm$ 1.1 times/300s *vs.* 2.3 $\pm$ 3.5 times /300s respectively; T=9, W=27, Wilcoxon-test, p=0.13; Fig. 4). Twelve (out of 16) of the singly-housed and eight (out of 16) of the family-housed males did not

show any aggression towards females (Fisher`s Exact test, p=0.27). We observed no sexual behaviors.

1.2.Response towards stimulus males vs. stimulus females

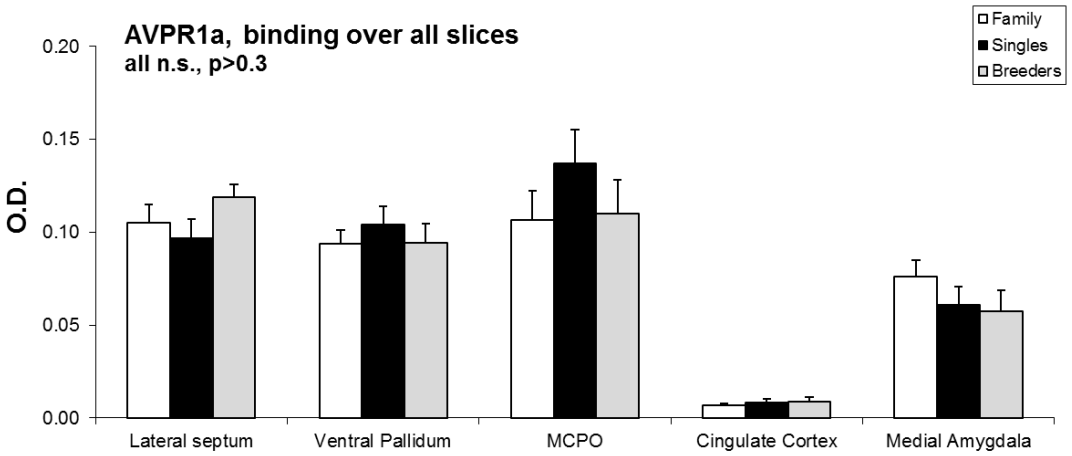
Family housed males did not groom the stimulus females significantly more often than they groomed stimulus males (T=20, W=5, Wilcoxon-test, p=0.82), but they spent significantly more time in bodily contact with the females (T=7, W=91, Wilcoxon-test, p=0.002), and they showed significantly less aggression towards the females (T=2, W=116, Wilcoxon-test, p=0.0002).

Singly housed males groomed the stimulus females significantly more often than they groomed stimulus males (T=14, W=63, Wilcoxon-test, p=0.03), and they showed significantly less aggression towards the females (T=8.5, W=38, Wilcoxon-test, p=0.049). The time they spent in bodily contact with the females did not differ from the time they spent in bodily contact with the stimulus males (T=38, W=60, Wilcoxon-test, p=0.13).

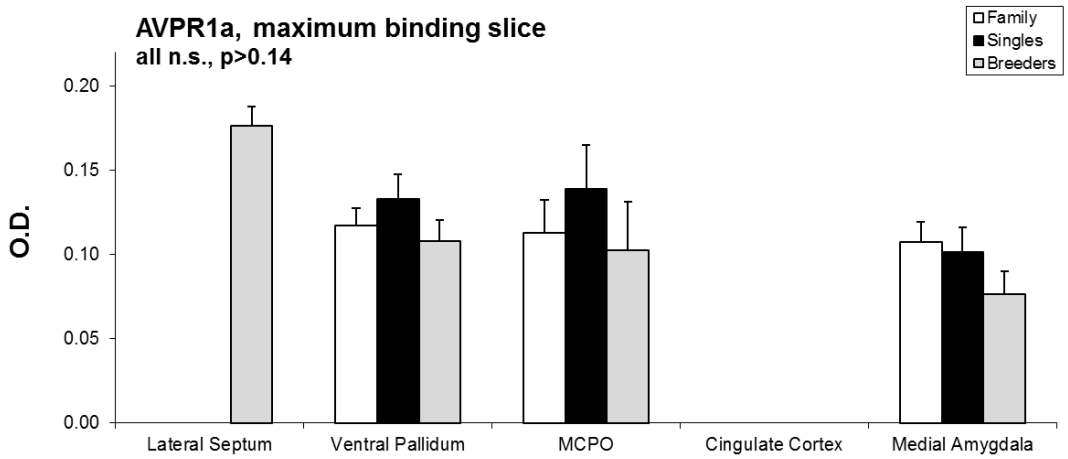
1.3.Differences in the arginine vasopressin receptor 1a when males were 10 weeks old

We first conducted paired tests between family and singly housed males for a maximal statistical power. When comparing the mean values of binding over all slides for each measured brain region, we did not find any differences between the two male tactics (all p>0.16; N=15 brother pairs). Similarly, when we compared the binding only for the slide with the maximum binding for each brain area, no significant differences were found between males living with their family and those that were singly housed (all p>0.12; N=15 brother pairs).

We then used unpaired tests to compare between all available samples including the brains from the 8 breeding males. When comparing the mean values of binding over all slides for each measured brain region, we did not find any differences between the three male tactics (Fig. 5a; all $p>0.3$). Similarly, when we compared the binding only for the brain slice with the maximum binding for each brain area, no significant differences were found between male tactics (Fig. 5b; all $p>0.14$).



a)



b)

Figure 5. Measurements of AVPR1a (uncalibrated optical density O.D.) in different brain areas of male striped mice representing three alternative tactics. No differences were found between tactics. a) Mean values over all slices in the specific brain areas. b) Maximum binding (single slice) for each brain area. There was no identifiable area of maximal binding for the cingulate cortex, which demonstrated very little specific binding. Mean and standard errors are shown.

Discussion

Our experiments induced significant differences in steroid hormone levels and in social behavior. Family housed males had significantly delayed sexual maturation and were characterized by high corticosterone but low testosterone levels when compared to singly housed males. These are the same differences as observed between philopatric and roaming males in the field [11]. Family housed males were also more aggressive and showed less pro-social behaviors. Interestingly, we did not find any evidence for differences in the expression of AVPR1a between family or singly housed males. Our hypothesis for future research is that the brains of males adopting the three different tactics are similarly responsive to circulating AVP, and that behavioral changes might thus be modulated by changes in AVP secretion, rather than at the receptor level.

Tactics describe the behavior shown by different individuals [1]. In species with fixed ARTs males following different tactics consistently differ in their behavior when tested under standardized conditions. For example in the midshipman fish (*Porichthys notatus*), only territorial but not sneaker males show territorial aggression and an acoustic mating call [26]. In tree lizards (*Urosaurus ornatus*), territorial males express much more aggression when tested under standardized conditions than do non-territorial males [2]. In species with plastic ARTs, males can switch between tactics depending on the environment and their own body condition [2, 27]. Our study is one of the first that shows in a species with plastic ARTs males using alternative tactics differ in their social behavior when tested under standardized laboratory conditions. Family living males showed more aggression and less pro-social behaviors when tested with strangers of both sexes than did their singly housed brothers. The low level of aggression observed in singly housed males is in agreement with the

assumption that roamers in the field are non-territorial [11]. Both family and singly housed males were less aggressive towards females than towards males, indicating that both types of males might have perceived females as potential mates.

Neuropeptides are strong modulators of social behavior and AVP can regulate social behavior by being secreted in different brain areas that are expressing AVPR1a [6, 7]. If the AVPR1a expression patterns differ between tactics then the brain's responsiveness to AVP will differ. In the present study we did not find evidence that male striped mice housed under different social conditions differ in the expression of AVPR1a in brain areas that are important in the regulation of social behaviors.

Roaming striped mice can switch to the breeder tactic within one day (Schradin, pers. observ.). A male switching from the roaming to the breeding tactic must be able to form social bonds with the females of the group into which it immigrates. Solitary striped mouse males have higher amounts of AVP stored in the PVN and BNST than breeding males [17], and the same trend has been found for a small sample of brains collected in the field. Thus, a roaming male encountering a group of communally breeding females into which he can immigrate could immediately secrete AVP. In the present study we did not find differences in AVPR1a expression between solitary and social males (Fig. 5), which could be due to small samples sizes, or which could indicate that the brains of solitary housed males might be responsive to such an AVP secretion, which could enable roamers to quickly form social bonds. At this stage, our data lead us to formulate a new hypothesis: it is rather AVP secretion than AVPR1a expression that might be important in the regulation of plastic ARTs. It would still be valuable to measure AVPR1a in free ranging males of the three tactics, as our captive study forced males into a specific tactic, while in the

field males can chose to remain either philopatric or to disperse and become solitary roamers.

Male prairie voles show three alternative reproductive tactics that are similar to the ones observed in striped mice: philopatric males, solitary wanderers representing roamers, and either monogamously or polygynously living males representing breeding males [9, 29]. Significant variation has been observed in microsatellite DNA associated with the gene avpr1a coding for the AVPR1a in prairie voles, which might explain variation in social behavior [30, 31]. However, while one study found long alleles to be associated with partner preference [31], another study found short alleles to be related to partner preference [30]. In the brain, the lateral septum and ventral pallidum are important for pair-bonding in prairie voles [32, 33], but wanderers and paired males do not differ in AVPR1a expression in these brain areas, but in brain areas involved in spatial memory. Genetic polymorphism in avpr1a does not seem to explain male ARTs in prairie voles [29, 35]Whether differences in AVP production / secretion exist between ARTs of prairie voles has so far not been investigated.

In the current study we demonstrated that singly housed males differ from their family housed brothers, expressing much higher testosterone levels while at the same time showing more pro-social and less aggressive behaviors. While this is in contrast to the theory predicting a causal effect of testosterone on aggression [36], it fits with the idea that testosterone could modulate anxiety in roaming striped mouse males [11, 37]. While the situation in our laboratory is much less complex than in the natural habitat of striped mice, our study indicates what future research would be important. Especially, our study indicates that the brains of males from the different tactics could be equally responsive towards AVP as in our study the AVPR1a did not

differ between treatments. This would allow changes in AVP secretion to modulate tactic changes. We urge researchers to consider AVP itself as a potential mediator for the expression of male ARTs in mammalian species with plastic tactics.

2. Acknowledgments

Funding was provided by the Swiss National Science Foundation (to CS) and the University of Zurich.

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